ROLE OF MATRIX STIFFNESS ON ADHESION, MIGRATION, PROLIFERATION AND DIFFERENTIATION OF HaCaT CELLS: AN *IN VITRO* STUDY

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By

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2014

CERTIFICATE

This is to certify that the research project report entitled **"Role of Matrix stiffness on adhesion, migration, proliferation and differentiation of HaCaT cells: An *In Vitro* study"** submitted by **Mr. Prerak Gupta** in partial fulfillment of the requirements for the award of the degree of Master of Technology in Biotechnology and Medical engineering with specialization in Biotechnology at the National Institute of Technology, Rourkela is an authentic work carried out by him under my supervision and guidance.

To the best of my knowledge, the matter embodied in the report has not been submitted to any other University/Institute for the award of any Degree or Diploma.

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ABSTRACT:

Since past few decades, a paradigm shift has been observed in the field of manual tuning of cell behavior using external mechanical cues. In this regard, matrix stiffness is considered as a crucial factor that regulates the cell adhesion, spreading, migration, proliferation and differentiation. The aim of this study was to decipher the role of matrix stiffness on key cellular processes including cell adhesion, spreading and cytoskeletal reorganization at molecular level. To investigate the aforesaid prospective, epithelial cells (HaCaT) were chosen and cultured on silicone based PDMS substrate within a stiffness range from 62 kPa to 855 kPa. Our result showed that extent of initial cell adhesion was higher on softer substrate whereas overall cell adhesion kinetics was faster for stiffer substrate. Cell proliferation was favored on the stiffer substrate as evident from MTT assay, pERK 1/2 expression and its nuclear localization. On the other hand rate of cell migration was higher on the softer substrates due to low E-cadherin expression and subsequent destabilization by β-catenin. Cellular differentiation was analyzed by checking filaggrin expression and our results indicate that stiffer substrate significantly favored the HaCaT cell differentiation. Modulation of such cell behaviors in response to matrix stiffness may prove to be useful for various tissue engineering purposes.

KEYWORDS: Matrix stiffness, stress relaxation, cell adhesion, cell migration

CHAPTER 1

INTRODUCTION & REVIEW OF LITERATURE

1. INTRODUCTION AND REVIEW OF LITERATURE

1.1 Mechanotransduction: A brief Review

The term "Mechanotransduction" is defined by the transmission of mechanical signal into chemical signals through some certain transducers present on the cell membrane, that enable the cell to sense its physical surroundings and respond accordingly. Any type of mechanical stress causes the activation of the mechanosensors such as sensitive ion channels and subsequent opening to produce a transduction current[1].

Initially it was believed that signaling pathways involved in the process of mechanotransduction are only important to maintain the structural integrity of mechanically strained tissues including cartilage, blood vessels, bone, and muscles but later a paradigm shift was observed when researchers started investigating various other cells types that are not exposed to mechanical stresses under physiological conditions like vascular smooth muscle cells, myocytes and endothelial cells. Stem cells fate also depends on external mechanical cues and can be directed towards particular cell lineage during differentiation in response to stiffness of the substrate on which cells are cultured and geometry of the surface. [2].

1.2 Cellular pathways involved in mechanotransduction

Mechanotransduction in diverse cell types is now a well known and established phenomenon and has been studied extensively. There are several biochemical signaling pathways that help cells to sense the external microenvironment. Primary transducers are integrin, stretched ion channels, and primary cilia. The principle interaction of cell with extracellular matrix is mediated through transmembrane integrin molecules. The intracellular domain of integrin is associated with other regulatory proteins and kinase that leads to activation of several downstream molecules including ERKs, Mitogen activated protein kinases (MAPKs), GTPases and other cytoskeletal proteins[3]. These integrin and its associated

proteins along with focal adhesion kinase are considered as a unit known as focal adhesion complex. It has been observed that focal adhesion complex is linked further to cytoskeleton actin through several adaptor proteins including talin, paxillin and vinculin and modulates the cell motility and proliferation capability [4]. Additionally, stretch ion channels mediate the mechanotransduction by controlling the calcium dependent pathways that subsequently regulates the cytoskeletal remodeling and other intracellular signaling. [5-6]. Another crucial factor that plays a role here is the presence of several growth factors and cell surface located G-protein coupled receptors[7], that are present outside of the cell and gets activated during mechanically stressed condition without the presence of specific ligand[8]. Non specific activation of cell surface receptors contributes to initiation of complex signaling pathways, hence mediating cellular mechanotransduction (Fig.1). To simplify this complexity and to understand the process of cellular mechanotransduction, a collective concept was proposed known as "tensigrity". This model clearly represents the transmission of mechanical forces in to biological systems by altering the structural arrangement of cytoskeleton leading to disruption of tensional integrity[9-10]. In order to regain this tensional integrity and maintain a mechanical homeostasis, a broad range intracellular and intercellular signaling pathways are triggered[11]. At a one step ahead, structural components connecting extracellular matrix to nucleus have also been explored. Occurrence of such connection suggests that these physical forces may also influence the intranuclear programming at gene level[12].

Another important thing that is noticeable here is the concept of cell traction forces. These forces are involved in generating the intracellular tension as cells are exposed to any mechanical stress[13]. These intracellular tension and other physical interactions determine vital key processes, including migration, contraction, cytoskeletal reorganization and adhesion [14]. This phenomenon becomes more significant for anchorage dependent cells, where physical interaction of cells with its matrix is vital for their survival. Also, this interaction establishes cell adhesion and spreading on extracellular

matrix that can be modulated by the application of biophysical factors. In other terms it may be conferred that adhesion of cells onto the matrix provides a platform to regulate the cellular behavior.

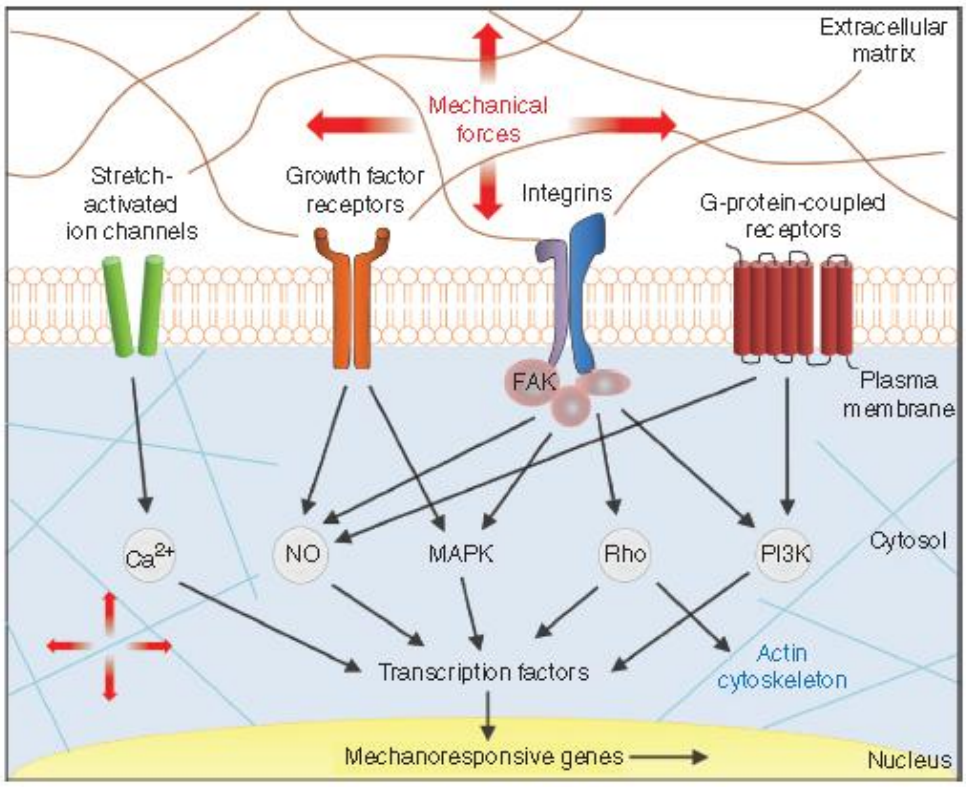


Figure 1: Intracellular signaling pathways involved in mechanotransduction. (adapted from Jaalouk and Lammerding, 2009)

1.3 Application of mechanotransduction in cellular engineering

It has now become a well known and established fact that various extracellular factors determine the cellular physiology and functioning. Primarily it was believed that only biochemical factors can determine the cell fate and same phenomenon was studied extensively. With time research has molded and researchers started considering on biophysical factors to program the cell destiny that plays a pivotal role by communicating through mechanotransduction signals. Wide range of cell lineages can transmit these signals and has been shown by various research groups. Mesenchymal stem cells (MSCs) are the precursor cells that have the capability to differentiate into various cell types of same lineage

including chondrocytes, osteoblasts, myocytes and adipocytes[15]. The determining factor that decides the direction of MSC differentiation towards a particular lineage is the matrix stiffness. If MSC are cultured on a soft substrate mimicking the stiffness of brain tissue tends to differentiate towards neuronal lineage. Similarly hard substrates of stiffness comparable with muscle favors myogenic lineage while hardest substrates of stiffness same as collagenous bone stimulates the osteoblastic differentiation of mesenchymal stem cells [16]. Additionally, a similar phenomenon was observed with embryonic stem cells where cells cultured on harder polydimethylsiloxane substrates upregulated early mesoderm markers and osteogenic differentiation[17].

Hair cells can also sense the surrounding mechanical microenvironment. This is usually generated by head movements and change in sound pressure that is sensed by the hair cells with good sensitivity. This mechanical signal is transmitted through the hair bundle that is highly expanded structure of stereocilia and arranged in rows with increasing height[18]. Vascular endothelial cells were found to be mechanoresponsive and shown to respond as a function of change in shear stress applied on them by blood flow under physiological conditions. Analysis of this mechanoresponsiveness at molecular level revealed the involvement of ERK1/2, AKT, and GSK-3beta mediated biological signaling. This signaling pathway leads to the activation of other downstream pathways [19].

Osteoblasts are well known mechanosensitive cells. Differentiation of osteoblast is usually triggered by various mechanical forces[20]. Within bone the presence of lacuna-canalicular system provides the base for cellular mechanosensing mediated through fluid flow strain. The mechanical loading of bone induces the extracellular fluid flow through lacuna-canalicular system that induces the bone cells through streaming potentials and wall shear stress. This streaming potential is generated by the movement of electrolytes driven by a pressure gradient across the channel[21]. *HR Wirtz et.al*. (1990) have reported the process of mechanosensing in alveolar type II cells. They found that a single stretch can cause increase in cytosolic Ca2+ concentration that stimulates the secretion of surfactants. Both of these responses were found to be dependent on magnitude of stretch stimulus. Cellular

response in a dose dependent manner is a clear indication that mechanical forces onto the non-muscle and non-neuronal cells may trigger various cellular events and may be a regulating factor for functioning of lungs[22]. All these reports suggests that most of the body cell types can sense the external mechanical cues and respond accordingly. Such kind of cellular behavior may prove significant for various issue engineering purposes where we need to manually regulate the cell fate according to the desired need or outcome.

1. 4 Diseases associated with mechanotransduction

Cellular ability to respond against changes in their physical environments is crucial for the maintenance and development of tissues like muscle and bone, which are exposed to various biophysical factors including shear stress, mechanical strain and cyclic strain. Several physiological processes that are vital for the entire organism like control of blood pressure and flow are also affected by this process. The process of mechanotransduction is now known to modulate the diverse cellular functions including cell adhesion, migration, proliferation, differentiation, protein synthesis, viability and apoptosis. It is now assumed that mechanotransduction is responsible for various diseases like cardiomyopathies and muscular dysfunction[23-24], osteoporosis [25], axial myopia and glaucoma [26-27], polycystic kidney disease [28], asthma and lung dysfunction [29-30], cancer [31] and central nervous system disorders [32]. It is also quite possible that even if the cellular mechanical processes working properly, that may still be a causative agent for several diseased pathological conditions. This pathway is valid in case where mechanotransduction is responsive in a dose dependent manner. For example, disturbance in fluid shear stress mainly at bifurcation point that usually triggers the vascular remodeling may result in atherosclerosis[33] or bone mass loss during the conditions of microgravity [25]. Both of these conditions are the result of altered levels of mechanical stresses that is mediated through normal transduction processes. Imbalanced stress levels cause upregulation or downregulation

of signaling pathways that leads to either over activation or lower activation of signaling molecules and as a consequence there occurs the breakdown of normal tissue function.

1.5 Matrix stiffness mediated mechanotransduction

1.5.1 Cells can sense the matrix stiffness via modulations in focal adhesions and cytoskeletal organization

Cells are able to sense the matrix stiffness via mechanical transducers. Primary force transducers includes stretch-mediated ion channels [34], primary cilia [35] and integrin [36]. Various research groups are involved in investigating the phenomenon of mechanosensing via substrate stiffness in variable cell types and have explored its presence in endothelial cells [37], smooth muscle cells [38], and transformed cells [39]. Most of these cells sense the substrate stiffness through contractility generated by cellular actin-myosin network that is carried out to extracellular region via transmembrane integrin protein receptors. These integrin after activation organize themselves into focal adhesions with the help of certain extracellular proteins and trigger intracellular signaling pathways. Activation of these pathways ultimately alters the cell substrate interaction, cytoskeletal reorganization and other regulating key cellular processes like proliferation, migration and differentiation. Modulation in cell behavior gives a clear indication towards cellular sensitivity for stiffness of substrate.

Investigation of fibroblasts and epithelial cells demonstrated that substrates of lower stiffness of 1 kPa tends to promote the formation of focal adhesions having dynamic irregularities [40]. On the other hand, elongated focal adhesions were found to be present in same set of cells cultured on substrates of higher stiffness of around 30-100 kPa. Also, tyrosine phosphorylation was found to be increased on focal adhesion kinase (FAK) and paxilin indicating that intracellular signaling is involved in stiffness mediated mechanosensing. Focal adhesions are present on cell membrane that mediates cell-substrate interaction. Alteration in structural arrangement and chemical modification of its intracellular

counterpart majorly influence this interaction. A linear correlation has been observed between these two parameters. Cell-substrate interaction is increased with increasing stiffness [41]. Other cellular properties are also influenced with modulation of stiffness. Working with endothelial cells, people have reported an increase in cell spreading area with increasing substrate stiffness [42]. An elongated spindle shaped structure of endothelial cells was observed on substrates of lower stiffness. Change in physical cellular morphology is known to modulate the cell behavior.

1.5.2 Matrix stiffness regulates cell-cell interaction, migration and proliferation

Substrate stiffness mediates cell–cell and cell-matrix interactions. Several research groups have tried to explore whether there occurs a relationship between substrate stiffness and these interactions and it was found that increase in substrate stiffness directly modulates cell-cell and cell-matrix interaction in terms of strength and prominence [43]. This relationship was found to be applicable with endothelial cells where it has been observed that endothelial cells cultured on compliant substrates prefers cell-cell interaction [44]. A similar relationship was also observed with epithelial cells where cells were found to be directed towards stiffer substrates. This directionality was later correlated with force generated and organization of actin cytoskeleton [45]. Matrix stiffness also influences the cell migration. Cell migration in response to external mechanical factors is usually termed as durataxis which was observed in fibroblasts that tends to migrate towards stiffer substrates [46]. Other cell lineages like smooth muscle cells shows the same phenomenon in terms of substrate rigidity gradient [47]. Implication of cell modulation in response to substrate stiffness is not limited since forces generated during cell-cell contact may also alternate the cellular proliferation. There might be the presence of a number of these interactions that regulates the cellular proliferation in a biphasic manner [48]. Cells present in a colony behave distinctly as compared to single cell. It has been observed that cells having at least one cell-cell interaction or cells present in contact with other cells are more proliferative than single cell. This concept is although not universally true since increasing colony size after a threshold

limit hinders the proliferation. Another interesting fact that is noticeable here is that later phenomenon is applicable because increasing cell to cell contact may reduce the ability of cell to adhere on the matrix. Reduction in cell-matrix interaction leads to reduced proliferative capability. This type of cell response is crucial to maintain the tissue integrity and function.

1.6 Skin structure and its mechanical properties:

The skin is the largest organ in human body. It plays various roles in our body. The main function of skin is to work as a barrier that separates internal organs of our body from external environment. The maintenance of its physical integrity and function even in presence of chemical, mechanical and other biological factors including temperature variation, exposure to harmful radiation and contact with pathogenic microorganisms shows its robustness. The same quality of skin has been evolved by several adaptations and structural modifications including its elasticity, toughness and tendency to repair quickly after any traumatic condition.

Mammalian skin mainly consists of two prominent layers, dermis and epidermis, that are separated by basement membrane. Dermis, that is the thicker layer, is mainly consisting of elastin and collagen type I protein that are majorly responsible for its elasticity and mechanical strength. Fibroblastic cells are dispersed throughout the dermal layer and regulate the accumulation and organization of fibrillar matrix. The thinner layer, called epidermis is multi layered and composed of polarized keratinocytes resting on underlying dermis. The polarized arrangement of keratinocytes provides directionality to the epidermal layer. Cells present in the lowest layer have the capability of dividing continuously throughout life of the organism and are in contact with basement membrane. This basal layer migrates upwards through the process called stratification which continues along with differentiation. .

Intact skin is under tensile stress continuously that can be evidenced by observing that introduction of a small wound causes the skin to relax and increases the wound diameter relatively.

This stress is known to be directional since it has been observed that circular wounds tend to elongate in the direction of higher stress. Mechanical properties of skin have also been studied extensively. The elastic modulus of the skin has shown to vary from 0.02 MPa to 57 [49]. To further investigate this large variation of elasticity, other factors including testing surface area, type of force applied, thickness of the skin and the level of skin hydration, have been considered. Also the location and positioning of skin under study influence this variability. Also the stiffness of forearm was measured and found to be around 210 kPa[50]. The extensively varying mechanical environment of the skin and its ability to maintain structural and functional integrity is an indication towards functional variation of keratinocytes under varying degree of mechanical environment.

Considering all facts, it becomes quite clear that sensing of mechanical microenvironment and alteration in cell behavior involves intracellular signaling pathways. Among all other, role of mechanical microenvironment in skin keratinocytes is yet to reveal in detail at molecular level. Here in this study, we tried to explore the role of matrix stiffness on the behavior of HaCaT cells in terms of cell-cell and cell-matrix interaction, differentiation, proliferation, rate of cell adhesion and migration.

Rationale of the work:

Since past few years more attention is being gained by cellular activity regulation in response to external mechanical cues. Researchers have proved that most of the body cell types are able to sense the physical stresses and this process is mediated by transmembrane regulators. Among other stresses, matrix stiffness may also influence the cellular behavior. Significant amount of research has been done focusing endothelial cells, osteoblasts, epithelial cells and muscle cells. In spite of all these advances the effect of matrix stiffness on keratinocytes has lately started to be explored. This is quite surprising since the structural integrity of the skin tissue is maintained by polarized layer of epithelial cells resting on the elastic surface i.e. basement membrane and dermis. So it becomes quite reasonable to investigate the effect of substrate stiffness onto the behavior of skin epithelial cells. Further, the major outcome of substrate stiffness has been explored but still investigation of these processes is limited at molecular level.

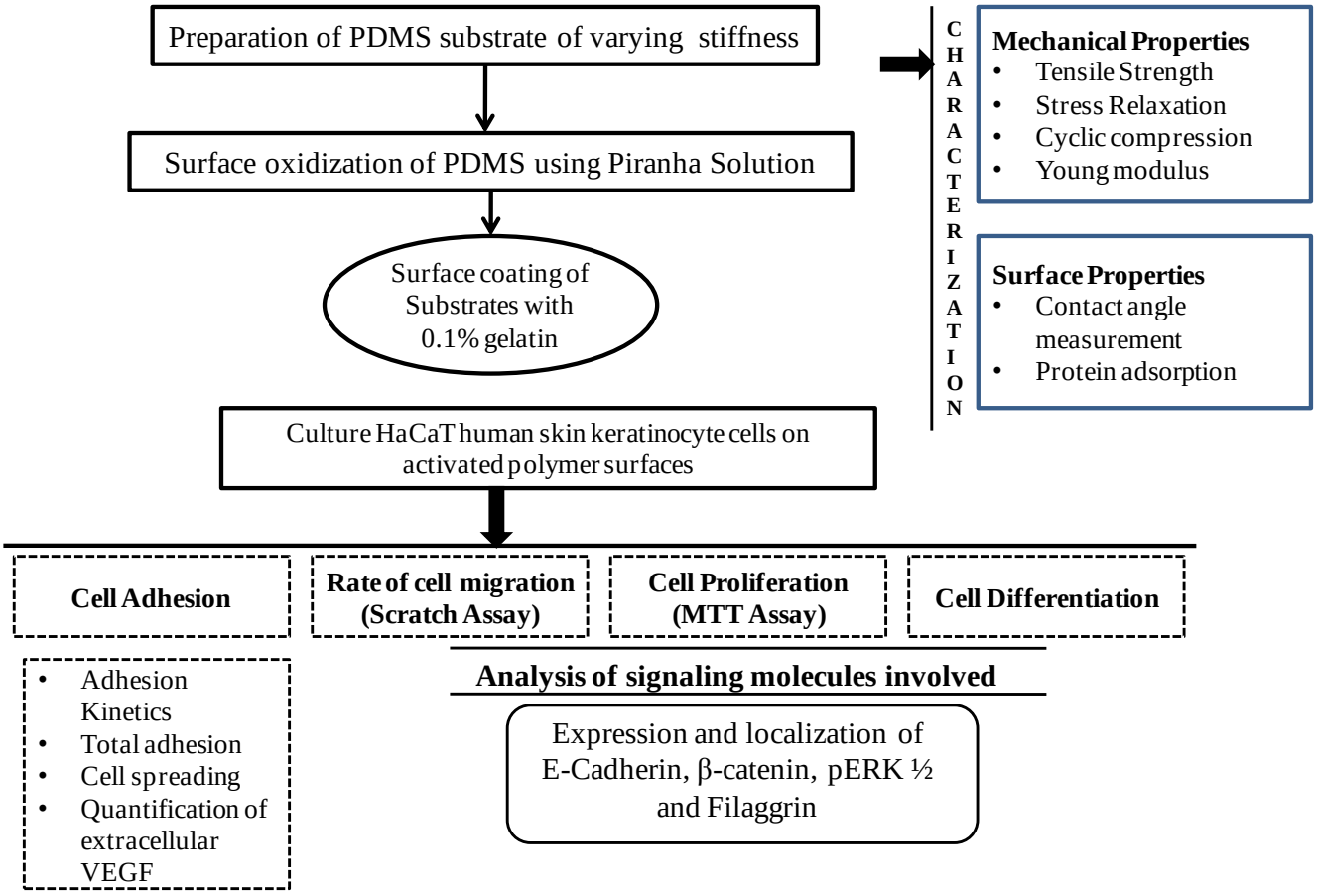
Considering the aforesaid perspective in mind we designed this study to explore the comprehensive behavior of HaCaT cells (skin keratinocytes) including cell adhesion, migration, proliferation and differentiation based on matrix stiffness as a complete behavioral response. We also tried to investigate the same responses at molecular level in order to get a clue of signaling molecules involved in differential behavior of HaCaT cells.

Structural arrangement of keratinocytes on basal layer makes them more suitable target for investigating the role of substrate rigidity on cell behavior. Significance of this type of investigation becomes physiologically relevant during wound healing, where keratinocyte cells continuously sense a change in substrate stiffness because of continuous stiffening of mature granulation tissue.

Objectives:

- To explore comprehensive behavior of HaCaT cells (skin keratinocytes) that includes including cell adhesion, migration, proliferation and differentiation in response to matrix stiffness
- To investigate the cellular signaling pathway that is involved in mechanotransduction of HaCat cells.

Workplan:



CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 Materials

SYLGARD (R) 184 SILICONE ELASTOMER KIT was purchased from Dow corning India Private Ltd. Hydrogen Peroxide 30%, Absolute Sulfuric Acid and Ethanol was bought from Merck, Mumbai, India. Isopropanol, Dulbecco's Minimal Essential Media (DMEM), Dulbecco's Phosphate Buffer Saline (DPBS), Trypsin-EDTA solution, Fetal Bovine Serum, Antibiotic-Antimycotic solution and MTT assay kit were purchased from Hi-media, Mumbai, India. The HaCaT cell line was procured from NCCS, Pune. Gelatin was obtained from sigma-aldrich.

2.2 Methods

2.2.1 Preparation of polydimethylsiloxane (PDMS) substrate and surface modification for cell culture

Substrate was prepared by mixing various proportions of PDMS and curing reagent as described by Goffin et. al.[51]. Three different ratios of PDMS: cross linker (w/w) viz. 10:1, 20:1 and 30:1, were used. Mixtures were than degassed by centrifugation at 5000g for 5 min and vacuum drying in a desiccator and baked at 65°C for 3.5 hours in dry hot air oven. Baked substrates were than treated with piranha solution (1:1 mixture of conc. Sulfuric acid and 30 % Hydrogen peroxide) for 10 min. Cell seeding was done on gelatin coated substrates for further experiments.

2.2.2 Mechanical Characterization

Prepared PDMS substrates were analyzed for tensile strength, cyclic compression and stress relaxation profile using TA.XT2i Texture Analyzer (Stable Micro Systems Ltd, Surrey, UK). Substrate stiffness was calculated in terms of young's modulus of deformation. (Table 1) Modulus values were selected in range from few to several hundred of KPa.

Table 1: Parameters used for mechanical analysis

Type of study	Type of fixture	Pre test speed	Test speed	Post test speed	Mode of study
Tensile Strength	3 mm probe	1 mm/sec	1.0 mm/sec	10 mm/sec	Tension(Force) (50g break sensitivity)
Stress Relaxation	3 mm probe	1 mm/sec	1 mm/sec	1 mm/sec	Auto (Force) (5g, 3mm)
Cyclic Compression	3 mm probe	1 mm/sec	1 mm/sec	1 mm/sec	Auto (Force) (3g, 8mm)

2.2.3 Protein Adsorption

Cell-matrix interaction is mediated through surface protein coating. Protein adsorption on oxidized and non-oxidized PDMS substrate surface was quantified using Bradford reagent by quantifying the unbound protein concentration. PDMS substrates of different base: cross linker ratios were prepared in a 96 well plate. BSA and lysozyme were used for protein adsorption study. Data was reported as relative adsorption.

2.2.4 Cell culture

Cells were maintained in Dulbecco's Modified Eagle Medium containing 10% FBS, 1% antibiotic-antimycotic solution at 37°C, 5%CO_2 in a humidified chamber. Cells were plated at least 24h before on substrate in optimum cell seeding density (~$1x10^4$ cells/ml) for any experimental study.

2.2.5 HaCaT cell morphology

After 24 hours of cell seeding, they were observed under phase contrast microscope (ZEISS Primo vert). For a closer observation of cell morphology, cells were fixed with 2.5% formaldehyde and dehydrated by ethanol treatment. Samples were than coated with gold particles for 90 sec under vacuum using sputter coating (Q150R-ES) and visualized under FEI NOVA NANO SEM 450.

2.2.6 Cell Adhesion kinetics

PDMS substrates were prepared in a 96 well plate and treated as described earlier. An optimum cell density i.e. ~1x10^4 cells were seeded per well at time t_0. Rate of cell adhesion on the substrates of varying stiffness was analysed by manual counting of non-adhered cells at t2, t4 and t6 hours using hemocytometer. Total cell adhesion was checked after 24 h. Percentage of adhered cells was calculated by using the following formula:

$$\% \textbf{ Cell Adhesion} = \frac{(\textbf{No. of cells seeded} - \textbf{No. of non adhered cells}) \times \textbf{100}}{\textbf{No. of cells seeded}}$$

2.2.7 Study of Cell Migration (Scratch assay)

HaCaT cells were seeded on PDMS substrates of varying stiffness in high cell density in a 12 well plate so that it reached confluency after 2 days. Wound was created onto the substrate surface in each well using 200µl pipette tip by making a scratch. Cells were washed with PBS and supplemented with fresh culture media. Cell migration was recorded and represented as rate of wound closure.

2.2.8 MTT assay for cell proliferation index

Same number of HaCaT cells were cultured in 96 well plate coated with surface oxidized PDMS of E_Y 855 KPa, 254 KPa and 62 KPa. MTT assay was done to analyze cell proliferation index at 24h, 48h and 72h time interval. Skin keratinocytes were incubated with 10% of MTT solution in DMEM for 4 h at 37°C in CO_2 incubator. Cultured media was removed and 200µl of dimethysulfoxide solution was added to dissolve the formazon crystals for 10 min at 37°C. The absorbance of the samples was recorded at 595 nm using an ELISA reader.

2.2.9 Cellular reorganization and Immunostaining

Cells seeded on surface modified PDMS substrates of varying stiffness were fixed after 2 days culture using 4% paraformaldehyde in PBS for 15 min. followed by permeabilization with PBST (0.1% Triton X-100 in PBS) for 15 min. Further blocking, primary antibody, secondary antibody and cytoskeletal staining was done as per recommended by manufacturer. For detection of immunofluorescence, stained cells were visualized using Olympus IX 81 confocal microscope using Fluoview1000 (Olympus) system.

2.2.10 Quantitative analysis of cell and nuclear spreading area

Spreading area of HaCaT cells on substrates of varying stiffness was quantified by processing the cytoskeleton and nucleus stained images using MacBiophotonics ImageJ software. Scale was set by converting the length of scale bar as per the known distance in to corresponding pixel size. Cellular and nuclear spreading area was calculated by manually enclosing the cell using freeform selection tool.

2.2.11 Quantification of secreted VEGF

The concentration of extracellularly secreted VEGF protein in cell supernatant was quantified. Cells were seeded in optimal cell density (~$1x10^5$ cells/well) in a 12 well plate coated with varying stiffness treated substrates. After 2 days HaCaT cell culture supernatant on substrates of varying stiffness was collected and analyzed using VEGF ELISA kit (abcam ab100662) after a brief spin in order to remove the cell debris and other particles. Same protocol was followed as per the manufacturer's recommendation.

CHAPTER 3

RESULTS & DISCUSSION

3. RESULTS AND DISCUSSION

3.1 Mechanical characterization of PDMS substrates

Mechanical behaviour of the prepared PDMS substrate was analyzed in terms of their tensile strength, stress relaxation and cyclic compression. Young's modulus of deformability of the substrates was obtained by processing the cyclic compression data. Mechanical characterization of all samples including tensile strength, stress relaxation and cyclic compression was obtained using TA.XT2i Texture Analyzer (Stable Micro Systems Ltd, Surrey, UK). For calculating the tensile strength of the substrates, rectangular strips of PDMS of dimension 5cm x 1 cm x 0.1 cm were prepared implementing an optimal manually designed mold for each substrate (Figure 2). Break sensitivity of the PDMS strips was recorded. (Figure 3)

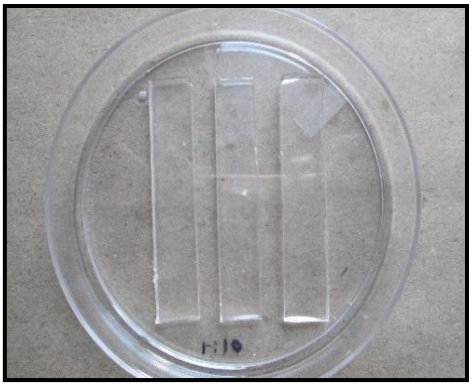


Figure 2 .PDMS strips prepared for tensile strength measurement

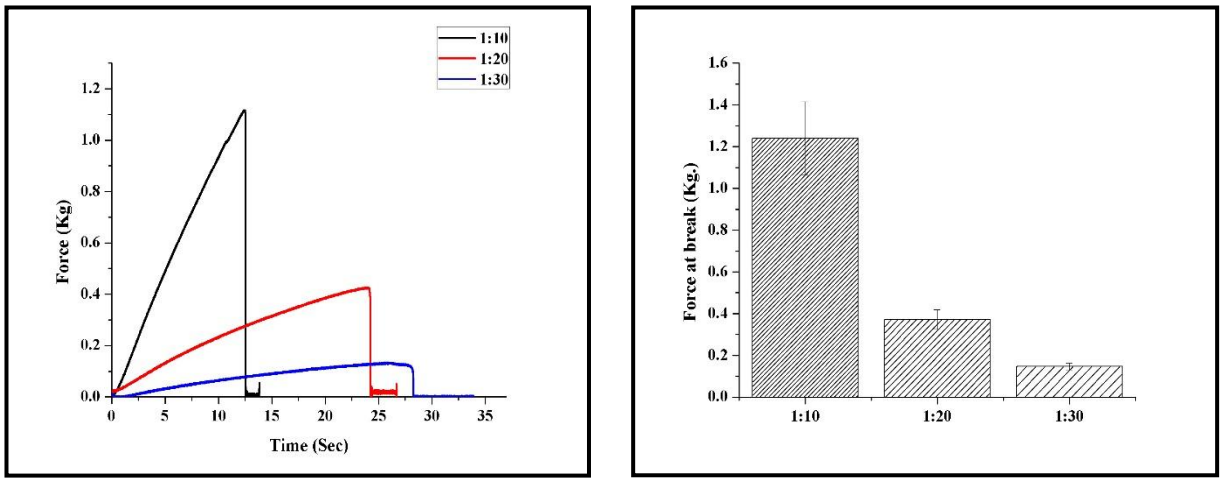


Figure 3 .Tensile strength analysis and corresponding break sensitivity of PDMS substrate

Substrate stiffness was obtained in terms of Young's modulus of deformability by processing data obtained for cyclic compression as per the protocol followed by *B. Otegbayo et.al (2007*) [52].Cyclic compression data for the prepared PDMS substrates was obtained for 2 cycles applying a trigger force of 3 g till 8 mm distance using auto (force) mode. The slope of the initial straight line portion of the force time curve (that represents the elastic region) indicates the stiffness or firmness of the material under study. This stiffness value is referred to as modulus of elasticity or Young's modulus in material engineering. Obtained Young's modulus value of all three PDMS ratios was found to be in the range of 62 kPa to 855kPa.(Table 2) These values cover a wide range of Young's modulus values and are physiologically relevant since it falls under the range of reported skin stiffness. More importantly, it has also been observed that the stiffness of mature granulation tissue at the later stages of wound healing varies from few hundred to thousands of kPa.[51] So it becomes quite reasonable to investigate the cell behavior under pathobiological conditions to make it clinically significant. Differential behavior of cells under the influence of substrate stiffness may prove to be key regulator for various tissue engineering prospects where we do need the manual tuning of cells fate as per the desired outcome.

Table 2 .Relation between PDMS: cross linker concentration and corresponding E_Y

Curing Agent: Base Ratio	Modulus of Deformation (kPa)
1:10	855.645
1:20	254.38
1:30	62.212

There also occurs a higher possibility that other intrinsic substrate properties including viscoelasticity may influence the cell behavior. This fact becomes significant because most of the adherent type cells adhere to the extracellular matrix mediated through focal adhesions. During this cell-matrix attachment cells exert a tangential tension onto the substrate mediated through actomyosin interaction and actin polymerization. This tension is referred as traction force. Recently *C.F.Soon*

et.al.2014 has reported shear sensitive liquid crystals for sensing the traction forces exerted by cells. These crystals were linear viscoelastic and non-toxic to the cells.[53]. Interpretation of stress relaxation of substrate was done by defining two forces. First is F0, which is force recorded after probe has moved a distance of 3 mm (after a trigger force of 5g). The probe was then allowed to stay at the same position for 30 s and resulting force was measured continuously. At the end of 30 s, second force was recorded, which was considered as F30. The % reduction of force from F0 to F30 is defined as %SR that was calculated using formula: [54]

$$\% \text{ SR} = \frac{\text{F0} - \text{F30}}{\text{F0}} \times 100$$

Where, F_0 = peak force at a target distance of 5 mm after a trigger force of 5 g and

F_{30} = final force after holding the probe for 30 sec.

The stress relaxation values of PDMS substrates were found to be increased as increasing substrate stiffness. Significant difference was recorded for all three PDMS: cross linker ratios ranging from 9.437 to 16.61 %. Results emphasized that at higher stiffness PDMS substrate shows greater molecular rearrangement properties as compared to lower stiffness within E_Y range between 850 kPa to 62 kPa.

3.2 Protein Adsorption

Cellular response to any mechanical stress depends on the connecting link between cell and surface. Here in this study we used gelatin as protein coat that gets adsorb onto the oxidized PDMS substrate either through hydrophobic or electrostatic interaction. This is the crucial step that provides a platform for cell contact with surrounding mechanical microenvironment and determines the cell response. It has already been shown that cell attachment, differentiation and proliferation is determined by the adsorbed protein's concentration, composition and conformation[55]. Therefore evaluation of protein adsorption is essential to evaluate the role of substrate stiffness on cellular behavior. Albumin is a

common body protein that is found in higher concentration. Hence negatively charged bovine serum albumin was used as model protein for adsorption study. Simultaneously another positively charged lysozyme was tested for adsorption on oxidized and unoxidized PDMS substrate.[56] Our results demonstrated that protein adsorption on oxidized surface was slightly higher as compared to un-oxidized surface. It may be inferred that surface with less hydrophobicity shows better adsorption of proteins. Additionally a non-significant pattern of BSA adsorption was found on all oxidized and unoxidized surfaces of PDMS with varying stiffness while on the contrary, an increased adsorption pattern of positively charged lysozyme on substrates with increased stiffness was observed. (Figure 6A & 6B) Such an adsorption pattern suggests the non specific surface binding of BSA. Presence of negatively charged silanol groups on the oxidized PDMS surface may influence the protein adsorption as shown by lysozyme, indicating higher silanol group density on surfaces with higher stiffness. Differential adsorption of protein on surface with varying stiffness indicates the possibility of altered cell behavior.

3.3 HaCaT Cell morphology on substrates of varying degree of stiffness

Chen et.al. has shown that cells with a flat surface survive better than round or less flattened cells.[57] Phase contrast images of HaCaT cells on silicone substrates evidenced the differential behaviour of cells under the influence of varying substrate stiffness. Cells cultured on the substrates with E_Y 855 kPa showed more spreaded morphology as compared to substrates of E_Y 62 kPa and corresponding cell perimeter was also higher on stiffer substrates. Cells seeded on substrate with E_Y 62 kPa showed a bright cell boundary confirming lower cellular spreading and adhesion at cell boundary. (Figure 7A) Unflattened morphology of HaCaT cells on lower stiffness substrates may be due to the presence of higher extracellular VEGF secreted. Here we can infer that low spreading area of cells on the lower stiffness substrates may be because of higher VEGF expression that tends to reduce the cell-plate adhesion.

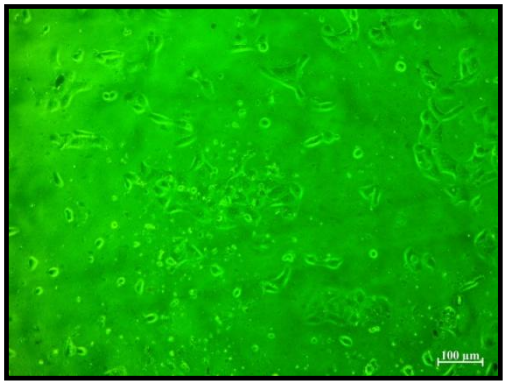


Figure 4: Representative image of HaCaT cell morphology seeded on PDMS substrate

Scanning Electron Micrograph of HaCaT cells on different stiffness substrates favoured the cell adhesion data where extent of cell adhesion was found to be higher on softer substrates at early stages. (Figure 8) Cells were found to respond against matrix stiffness as cells organize themselves distinctly. Stiffer substrates favoured cell spreading whereas almost round shape morphology was observed on substrates of lower stiffness. Presence of small white dots on substrate with E_y =62 kPa, may represent the extent of cellular reorganization where cells rearrange their morphology as per the substrate stiffness.[58] Such kind of cell behaviour assured that HaCaT cells also respond as per their mechanical microenvironment. This differential cell behaviour under the influence of substrate stiffness may play a crucial role in determining cell viability.

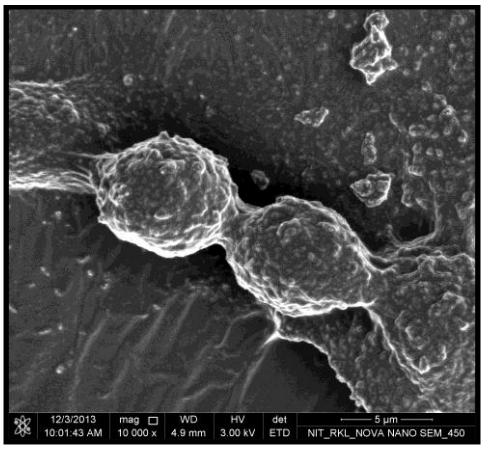


Figure 5 .Representative image of FESEM micrograph of HaCaT cells seeded on PDMS substrate

3.4 Extent of cellular reorganization

In order to record cellular response quantitatively as a function of matrix stiffness, here in this study we tried to calculate the area of cell spreading along with ratio of major to minor axis, that may give an idea about extent of cytoskeletal rearrangement. For such type of data acquisition it becomes pre-requisite to visualize the cell cytoskeleton that was attained by staining the cell cytoskeleton and nucleus with fluorescent dyes and processed the acquired images. (Figure 9) Data obtained after processing the images revealed that HaCat cells tends to spread comparatively more on stiffer substrates (E_Y= 855kPa) while it reduced significantly for substrates with E_Y = 62kPa . Cell circularity factor was close to 1 on substrate with E_Y = 62kPa. (Figure 10A)These results supported FESEM data where almost round cells were observed. On the other hand, nuclear area and circularity factor did not show any significant difference on substrates of varying stiffness. (Figure 9) Alteration in cell spreading area as function of matrix stiffness is crucial where we need to tune the cell behavior manually.

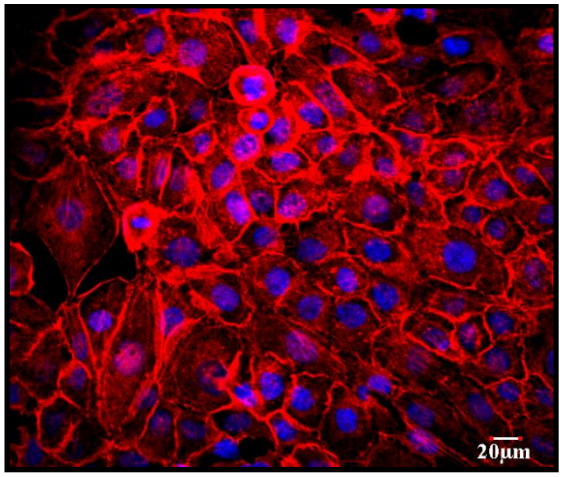


Figure 6 .Representative image of cell Spreading cultured on PDMS substrate.

3.5 Cell adhesion kinetics and extent of cell adhesion

From biological perspective, cell adhesion is a crucial step for survival and functioning of adherent

cells.[59] Most of mammalian cells are of adherent type so they require a solid surface for attachment and consequent survival because of which the initial steps of cell-matrix attachment are important. In tissue engineering, cell adhesion to a surface is critical because adhesion proceeds other events, such as cellular spreading, migration, and proliferation. Cell adhesion on its substrate is usually a biphasic process that involves both specific ligand-receptor and non-specific interactions.[60] Such interactions not only help in physical attachment but also are the basis for cell and matrix communication that helps cell to sense its microenvironment. Depending on matrix stiffness cells respond distinctly. Here in this study, we found that rate of cell adhesion with time varies depending on matrix stiffness. A comparatively faster rate was observed on stiffer substrates while lower stiffness surfaces favoured a slower and plateau pattern. Consequently on the other hand, total number of cells adhered after 24 h were found to be approximately same. Such a cellular response might be as a result of sticky nature of lower stiffness substrates. Higher extent of cell adhesion on lower stiffness substrates within first few hours may be physiologically significant during metastasis.

3.6 Matrix stiffness regulates the cell migration

The effect of substrate stiffness is more significant on single cells as compared to whole cell sheet. Various research groups have demonstrated that Increasing substrate stiffness promotes the migration of epithelial cells and fibroblasts from cell colonies in vitro.[43, 61] Our scratch test results showed that the migration of HaCaT cells was faster on the stiffer substrate during first four days while a sudden increase in migration rate was observed on lower stiffness substrates after completion of four days. Initially the fastest migration was observed on the silicone substrate with E_Y = 855 kPa While net migration was more prominent on the substrates with E_Y = 62 kPa. (Figure 7) This indicates that during initial days of culture, the epidermal cells preferred a rigid substrate; it also indicates a connection to cell mechanotransductive pathways. Since cells are known to be anchored on substrates through integrin mediated focal adhesions, it becomes the most probable mediator through which

mechanoresponsive pathway gets initiated. This mechanosensing is mediated through integrin activation that leads their clustering[62-63]. Tension may also initiate the activation of integrin[64]. Stiffer substrates activate integrins to promote formation of focal adhesions, and subsequently initiates Rho/ROCK pathway and increases cell migration and contractility[3, 65].[66] Clustering and consequent activation of integrin molecules are connected to cytoskeletal actin fibers through e-cadherin that has been shown to mediate the matrix stiffness mediated regulation of collective cell migration [67]. Expression profile of E-cadherin demonstrated higher expression in 855 kPa substrates which was found to be localized throughout the cytoplasm while on the lower stiffness substrates, E-cadherin expression was comparatively lower that causes lower degree of actomyosin contraction during collective migration. (Figure 8B) Membrane bound E-cadherin is known to be stabilized by β-catenin that may be localized throughout the cell. Our results suggested the membrane localization of β-catenin in cells cultured on substrates of varying stiffness but degree of expression was found to be favored by 855 kPa substrates. (Figure 8A) Presence of these two substrates onto the membrane helps in collective cell migration. Cells tend to migrate away from one another if physical signals from the substrates are stronger than those from cell– cell interactions, and towards one another if cell–cell interactions provide a stronger mechanical input[68] Our results suggested that after ~6 days substrates with E_Y 62 kPa provides stronger cell to matrix signals that helps cell to migrate away, hence faster migration rate. Cytokines are also important regulators of proliferation and differentiation of keratinocytes. Imbalance of these cytokines may cause the skin inflammatory disorders[69]. In normal human skin, VEGF is known to be expressed and secreted by keratinocytes. During diseased conditions like psoriasis, an increase in VEGF secretion has been observed [70]. VEGF has shown to increase cell-cell interaction while it decreases cell-plate interaction. [71] VEGF secretion levels in HaCaT cells have been reported minimum in comparison to squamous and facial epithelial cells. [72] Here in this study we found that cells cultured on substrates with E_Y 62 kPa produced highest amount of VEGF as compared to higher stiffness substrates. To reduce the ambiguity of VEGF secretion due

to difference in cell number, total cell viability in each sample was quantified using MTT assay. Results were reported as relative VEGF expression index.

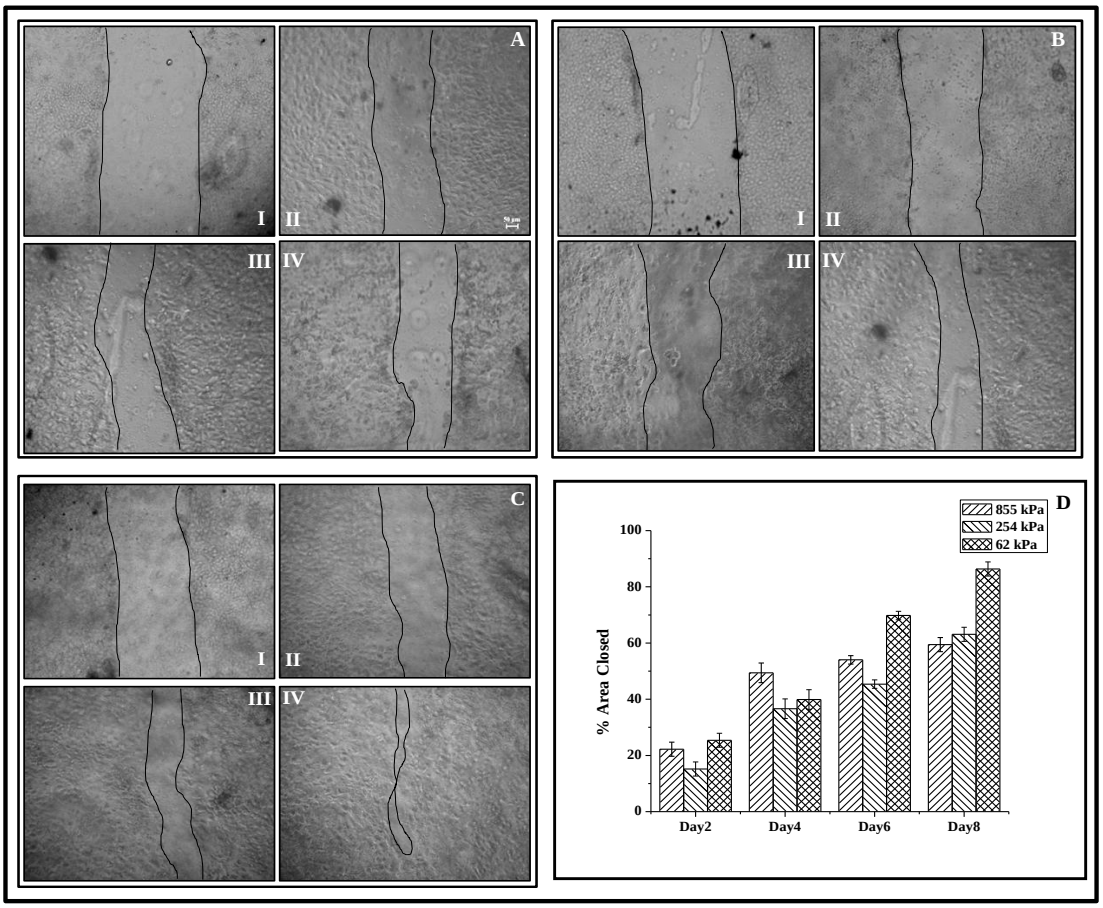
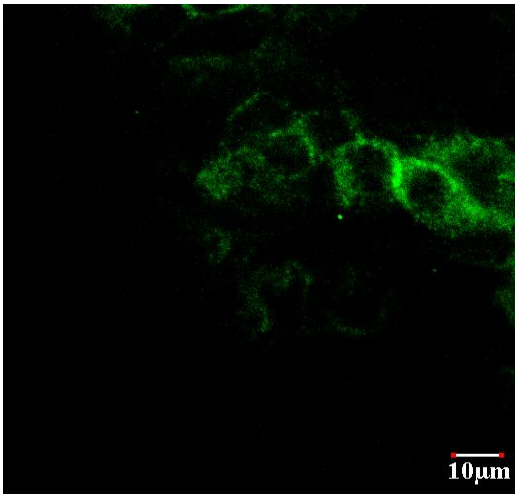
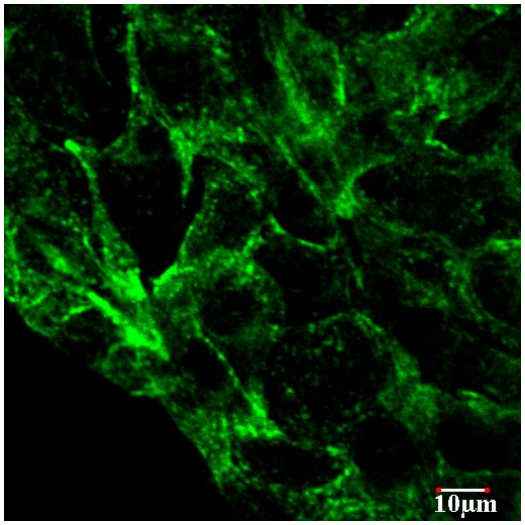


Figure 7 .The migration of epidermal cells on silicone substrate with E_Y = 855 kPa (A), E_Y = 254 kPa (B), and E_Y = 62 kPa (C) after day 2 (I), day 4 (II), day 6 (III) and day 8 (IV). The migration rate of epidermal cells on the silicone substrate with E_Y = 855 kPa, 254 kPa, and 62 kPa after 2, 4, 6 and 8 days. (D).



(A)



(B)

Figure 8 .Representative images of expression profile of, (A) β-catenin and (B) E-Cadherin cultured on PDMS substrates

3.7 Cellular Proliferation

Cellular proliferation is usually dependent on several factors. Proper adhesion of adherent cells onto the substrates apparently regulates the cell dividing ability. Surface area of contact between cell and matrix has also shown to determine the cellular activity. [57] Results of MTT assay demonstrated that cellular proliferation was occurred throughout the 3 days of culture period and HaCaT cells show more

activity on substrate with higher stiffness with E_Y 855kPa. Significant difference was observed with lower stiffness substrates where cell activity was low. Data was recorded till day 3, a similar trend was found to be followed. Data obtained was outcome of three independent experiments. Results are reported as mean ± SD. Our results inferred that cell ability of proliferation increases with increase in substrate stiffness. To further verify and investigate the cell proliferation we studied the expression of pERK ½. Phosphorylation of ERK leads it towards nuclear localization where it helps cells to pass through G1 stage and enter to the S phase of cell cycle. Without nuclear localization it would not transmit downstream signal that may lead to cell cycle arrest in G1 phase. Also an overexpression of ERK leads to hinder the cell growth. Our results showed the nuclear localization of pERK in all substrate seeded cells but cells cultured on substrates of higher stiffness showed a higher level of expression (Figure 9), supporting the MTT data.

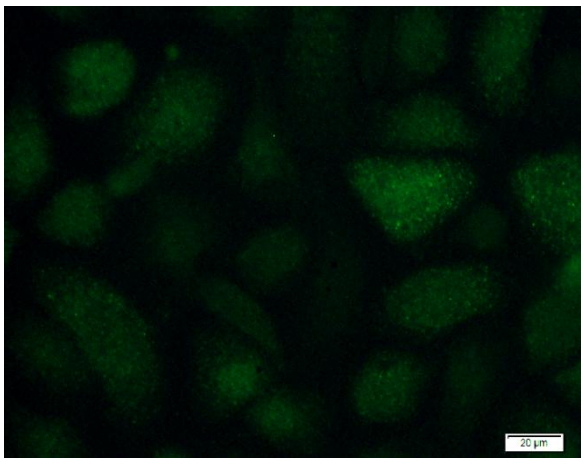


Figure 9. Representative Image of expression profile of pERK 1/2 in HaCaT cells seeded on PDMS substrate

3.8 Diffrentiation of HaCaT cells

Late differentiation marker, filaggrin was used to investigate the role of matrix stiffness on HaCaT cell differentiation. It is a filament associated protein which binds to cytoskeletal keratin intermediate fibres in epithelial cells. Filaggrin also regulates homeostasis of epidermal cells. Filaggrin primarily is

found in the cells of stratum corneum in inactivated state known as profilaggrin. Upon activation profillagrin is phosphorylated and cleaved in fillagrin monomers that tend to incorporate in to lipid membrane and maintains skin barrier function. It also releases free amino acids that help in retention of water molecules maintaining hydration level[73]. Substrate of 855 kPa favored the filaggrin expression while significant lower levels were found to be expressed after 21 days culture time on 62 kPa substrates. Its distribution was found to be uniform throughout the cell. (Figure 10)

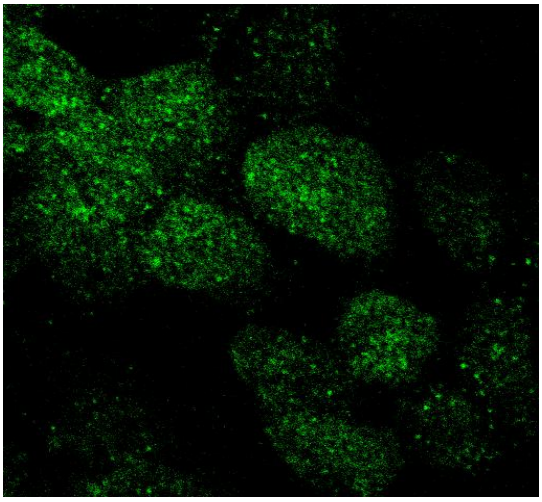


Figure 10 .Representative image of filaggrin expression in HaCaT cells seeded on PDMS substrate

CHAPTER 4

CONCLUSION

4.0 CONCLUSION

Manual tuning of cellular behaviour is clinically important. Various mechanical stresses are known to modulate the cell behaviour. In the current study, we found a significant difference in cell behaviour under the influence of matrix stiffness. Stiffer substrate was found to favour the HaCaT cell proliferation and late differentiation while migration was favoured on lower stiffness substrates. Such response may prove useful during metastasis where cell-matrix contact is either weakened or lost. Our study showing different cell adhesion response as a function of matrix stiffness is a positive indication towards manual tuning of cell fate that may prove useful during various tissue engineering applications.

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